

# CYANOBACTERIAL NUCLEIC ACID FRAGMENTS ENCODING PROTEINS USEFUL FOR CONTROLLING PLANT TRAITS VIA NUCLEAR OR PLASTOME TRANSFORMATION

## FIELD OF THE INVENTION

The present invention relates to improved screening methods for identifying and utilizing cyanobacterial genes for modifying plant traits, and to cyanobacteria as an alternative source of *ahas* and *pds* genes for plant transformations, in particular genes encoding herbicide insensitive proteins, and elements of genes for control of expression in plastids.

## RELATED APPLICATION

This application claims priority from Provisional Application Serial No. 60/214,705, filed June 27, 2000.

## BACKGROUND OF THE INVENTION

Cyanobacteria are considered to be the precursor of plant chloroplasts. Cyanobacteria possess all beneficial features of prokaryotes like ease of handling, rapid growth under defined conditions, availability of replica plating techniques, easy genetic manipulation by mutagenesis or transformation and availability of established mutants. Cyanobacterial metabolism also share important features with higher plant metabolism such as oxygenic photosynthesis by two photosystems and autotrophy with respect to reduced nitrogen, sulfur and carbon dioxide. Therefore, efficacy of compounds in cyanobacteria can be indicative of similar performance in higher plants.

The photosynthetic membranes of cyanobacteria, plants and algae contain essential pigments called carotenoids, which function in protecting chlorophyll against photo oxidative damage by singlet oxygen, as well as acting as accessory pigments in photosynthetic light harvesting. These carotenoids are also precursors of vitamin A in human and abscissic acid in plants. The first committed step in carotenoids biosynthesis is the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to yield the colorless phytoene. Desaturation of phytoene through the insertion of four double bonds gives rise to lycopene, and further cyclization reactions lead to the generation of  $\beta$ -carotene. Phytoene desaturase(pds) mediates the first two steps of desaturation of phytoene, disruption of which results in an observable bleaching symptom. As such, a number of commercial herbicides directed at inhibiting this enzyme have been developed, *e.g.* norflurazon, fluridone, and fluorochloridone.

In addition, as ancestral precursors to chloroplasts, cyanobacterial genes share features common to chloroplast genes. Gene elements, such as promoters, ribosome binding sites, etc. are similar and can be cross-functional between chloroplast and cyanobacteria. Therefore cyanobacterial genes make ideal candidates for plastome targeted transformation, and in particular chloroplast transformation.

There are a number of references in the literature to screening methods and assays utilizing cyanobacteria. These include methods using cyanobacteria for the screening of compounds to identify inhibitors of specific metabolic pathways and identification of novel herbicidal modes of action. [Hirschberg et al, 1996] describes an *Erwinia* gene transformed into host cells selected of cyanobacteria, specifically *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803, which was used as a screen for beta-carotene biosynthesis and for mutants resistant to herbicides specifically bleaching herbicides of the trialkylamine family. The screening for bleaching activity is described by [Sandmann et al, 1991] as a means to discover new herbicides with different core structures which inhibit phytoene desaturase (pds), a membrane bound enzyme in the carotenogenic pathway catalyzing the hydrogen

abstraction step at the first C40 precursor of beta-carotene. [Windhoevel et al, 1994]

describes a screen involving genes coding for *pds* of the non-photosynthetic bacterium

*Erwinia uredovora* introduced into the cyanobacterium *Synechococcus* as a convenient

experimental model for higher plant transformation and resistance to herbicides. The

5 functionality of the heterologously expressed phytoene desaturase in the transformants was

demonstrated in assays. Other references such as [Babczinski et al, 1995] identify new

herbicide class inhibiting *pds* based on a screen utilizing the unicellular cyanobacteria

*Anacystis*. [Chamowitz et al, 1993] described a cell-free carotegenic assay to identify

herbicide resistant algal *pds* mutants. Inhibition of carotenoid biosynthesis by herbicidal m-

10 phenoxybenzamide derivatives was investigated in a cell-free in vitro assay using the

cyanobacteria *Aphanocapsa* by [Clarke et al, 1985], and subsequently by [Kowalczyk-

Schroeder et al, 1992]. [Sandmann et al, 1991], describes a non-radioactive cell-free assay to

quantitatively determine inhibition of plant-type *pds* by bleaching herbicides. They further

developed a cyanobacterial *pds* assay system, a mode of action assay utilizing the

15 cyanobacteria *Anacystis*, and assays using algal cells. The present invention, however, differs

by identifying improvements to the current screening methods and assays, and uses these

improvements to identify novel nucleic acid fragments having herbicide resistance mutations

in the *pds* gene.

The prokaryotic acetolactate synthase (*ahas*) enzyme exists as two distinct, but

20 physically associated, protein subunits. In prokaryotes, the two polypeptides, a "large

subunit" and a "small subunit" are expressed from separate genes. Three major *ahas*

enzymes, designated I, II, III, all having large and small subunits have been identified in

enteric bacteria. In prokaryotes, the *ahas* enzyme has been shown to be a regulatory enzyme

in the branched amino acid biosynthetic pathway [Mifflin et al, 1971], and only the large

25 subunit has been observed as having catalytic activity. From studies of *ahas* enzymes from

microbial systems, two roles have been described for the small subunit: 1) the small subunit is

involved in the allosteric feedback inhibition of the catalytic large subunit when in the

presence of isoleucine, leucine or valine or combinations thereof; and 2) the small subunit enhances the activity of the large subunit in the absence of isoleucine, leucine or valine. The small subunit has also been shown to increase the stability of the active conformation of the large subunit. The expression of the small subunit can also increase the expression of the large subunit as seen for AHAS I from *E.coli* [Weinstock et al., 1992].

The *ahas* large subunit protein has been identified in plants, and has also been isolated and used to transform plants. An *ahas* mutant allele isotype of the *ahas* III large subunit protein, having the tryptophan at position 557 replaced with leucine has been found in a *Brassica napus* cell line [Hattori et al., 1995]. The mutant protein product of this gene confers sulfonylurea, imidazolinone and triazopyridine resistance to the cell line. This mutant allele, when expressed in transgenic plants, also confers resistance to these herbicides.

Until recently, there was no direct evidence that a small subunit protein of *ahas* existed in eukaryotic organisms. Recently, other groups, through the use of Expressed Sequence Tags (ESTs), have identified sequences homologous to the microbial *ahas* small subunit genes in an eukaryote, the plant *Arabidopsis*. These groups showed that a randomly isolated *Arabidopsis* cDNA sequence had sequence homology with the *ahas* small subunit sequences from microbial systems. Since then, ESTs from small subunit genes have been described from other eukaryotes such as yeast and red algae. [Duggleby et al, 1997] describes three EST sequences, two from *Arabidopsis* and one from rice, that have homology to known prokaryotic small subunit cDNA sequence from *P. purpurea*.

Several references to *ahas* screens and assays utilizing cyanobacteria exist in the prior art. [Powell et al, 1990], reported on the role of cyanobacteria for herbicide screening but no mention was made of the *ahas* “small subunit” identified in our invention. They reported that our understanding of the mode of action of certain herbicides which inhibit photosynthesis has been facilitated by studies with cyanobacteria. In the case of sulfonylurea herbicides which inhibit branched chain amino acid biosynthesis, the resistance shown by a cyanobacterium is due to an insensitive acetolactate synthase enzyme. These studies are not

consistent with the results reported by Freiburg et al. discussed below, in which the cyanobacterial gene is sensitive. If other insensitive target enzymes were to be found, cyanobacteria could be useful sources of genes for the cloning of herbicide resistance into higher plants. They presented data showing high levels of resistance of certain cyanobacteria to glyphosate, an inhibitor of aromatic amino acid biosynthesis. [Dunahay et al, 1997], discloses a method to transform chlorophyll containing *algae*, which includes introducing a recombinant molecule comprising a nucleic acid molecule encoding a dominant selectable marker operatively linked to an *algal* regulatory control sequence into the chlorophyll C-containing *algae*. However, unlike our invention, the mutant *ahas* was introduced into *algae*, not *cyanophyceae*, to detect inhibitors.

WO 98/06862 (Calgene) discloses plants transformed with the *Erwinia* phytoene desaturase gene for altered carotenoid levels and fatty acid. JP 6,343,476 (Kirin Brewery) describes the production of bleaching herbicide-resistant plants by transformation with the *Erwinia* *pds* gene. WO 98/06862 (Zeneca) discloses transgenic plants resistant to many classes of herbicides but the source of the genes, whether *pds* or *ahas* or from *Synechocystis* is unspecified. Also, U.S. 5,378,824 (Dupont) and U.S. 5,661,017 (Dunahay et al.) both report the transformation of a plant *ahas* gene, not a *Synechocystis* gene, into a number of *phyla* and classes including *algae*.

Freiburg et al, 1990, reported on herbicide resistant *Synechococcus* *ahas* gene expressed in *E. coli*. The report describes the isolation and molecular characterization of *acetolactate synthase* genes from the sulfonylurea-sensitive enzyme and from the sulfonylurea-resistant mutant, which specifies the enzyme resistant to sulfonylurea herbicides. The ALS gene was cloned and mapped by complementation of an *E. coli* *ilv* auxotroph that requires branched-chain amino acids for growth and lacks ALS activity. The cyanobacterial gene is efficiently expressed in this heterologous host. The resistant phenotype is a consequence of proline to serine substitution in residue 114 of the deduced amino acid sequence. Functional expression of the mutant gene in *Synechococcus* and in *E. coli*

confirmed that this amino acid sequence is responsible for the resistance. [Linden et al, 1990], reported cyanobacteria *Synechococcus* PCC 7942 mutants selected against the bleaching herbicide norflurazon. One strain exhibited cross-resistance against another bleaching herbicide fluorochloridone, but the other three strains did not show cross-resistance against other phytoene desaturase (*pds*) inhibitors. [Sandman et al, 1991] reported on mutants from *Synechococcus* PCC 7942, which were selected for tolerance to various bleaching herbicides. A mutant NFZ4 established a high degree of cross-resistance to both norflurazon and fluorochloridone, but not to fluridone. [Chamowitz et al, 1991] cloned and sequenced a *pds* gene from the cyanobacteria *Synechococcus* PCC 7942, also resistant to the bleaching herbicide norflurazon. The identified mutant is a Val  $\Rightarrow$  Gly change at position 403 in the *Synechococcus*, but not *Synechocystis* *pds* protein. [Sandmann et al, 1998] reported bacterial and fungal *pds* as a target for bleaching herbicides, and discussed the identification of cyanobacterial mutants with resistance to specific compounds and their cross-resistance to other bleaching herbicides.

Cyanobacteria *Synechocystis* was originally described in Vioque et al, 1992. A spontaneous mutant, strain AV4, which is resistant to norflurazon, was isolated from *Synechocystis* PC 6803. DNA isolated from the mutant AV4 can transform wild-type cells to norflurazon resistance with high frequency. Sequence analysis of the clone identified an open reading frame that is highly homologous to the previously sequenced *pds* genes from *Synechococcus* and soybean. In both cyanobacteria and plants the *pds* gene is highly conserved: the *Synechocystis* PCC 6803 *pds* gene is 82% and 61% identical to the *Synechococcus* PCC 7942 and the soybean *pds* genes respectively. [Sandmann et al, 1994] identified three distinct *Synechocystis* mutants selected against norflurazon, and showed modification of the same amino-acid of phytoene desaturase into three different ones. In all cases, the same amino-acid Arg<sup>195</sup> was modified either into Cys, Pro or Ser. The degree of resistance was highest when Arg was changed into Ser.

While the literature has several references to *pds* herbicide resistant transgenic plants, our intervention exemplifies improvements to current cyanobacteria screening methods. Our improvement has identified novel nucleic acid fragments from *Synechocystis* PCC 6803. The mutant *pds* (phytoene desaturase) gene and *ahas* large and small subunits are useful in the identification of novel *pds* and *ahas* inhibitors and, in plant transformations for conferring resistance and cross-resistance to certain bleaching herbicides and AHAS-inhibiting herbicides.

## SUMMARY OF THE INVENTION

Therefore, the present invention improves the current cyanobacteria screening methods. Our improvement has, in turn, identified novel nucleic acid fragments from the cyanobacterial *Synechocystis* PCC 6803. The mutant *pds* (phytoene desaturase) gene and *ahas* (Acetohydroxyacid synthase) large and small subunits are useful in the identification of novel *pds* and *ahas* inhibitors and, in plant transformations for conferring resistance and cross-resistance to certain bleaching herbicides and imidazolinones.

Specifically, screening methods were used for identification of novel *Synechocystis* mutations that provide resistance to 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide, an inhibitor of *pds*.

The identification of a novel mutation in the *pds* gene together with the fact that this gene is highly homologous between cyanobacteria and plants, will aid our efforts in engineering crops for resistance to herbicides through the introduction of site-directed mutation in the target *pds* gene.

Novel mutations displaying unique resistance to 4'-fluoro-6[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide will aid in programs of engineering crops for resistance to 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide and potentially other *pds* inhibiting herbicides via chloroplast-mediated transformation. Alternatively, mutant

forms of *pds* genes with mutation(s) at position(s) similar to the *Synechocystis* gene can be obtained for any given crop species, and used further for genetic transformation. The identification of additional novel mutations conferring resistance to 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide should shed light on the structure of 4'-fluoro-6-[(alpha,alpha,alpha, trifluoro-m-tolyl)oxy]- picolinamide binding site, and serve as a valuable guide for designing novel inhibitors of this enzyme.

Therefore, in preferred embodiments, the present invention provides novel *Synechocystis* mutant *pds* gene(s) conferring resistance to 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide.

Also, in further preferred embodiments, the present invention provides a method of using a simple genetic system, *Synechocystis*, to select and isolate mutants forms resistant to 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]- picolinamide.

In additional embodiments, a method for the preparation of *pds* resistant nucleic acid fragments from the cyanobacteria *Synechocystis* EMS resistant cell lines is provided.

In additional preferred embodiments, the present invention provides novel *Synechocystis* mutant *pds* gene(s) conferring cross-resistance to known PDS inhibitors and 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]- picolinamide.

Another approach to the use of cyanobacterial genes for controlling plant traits is to use natural cyanobacterial genes, which already have desired characteristics. The acetolactate synthase (AHAS) enzymes from *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 are naturally resistant to imidazolinone and other AHAS inhibiting enzymes. The AHAS genes from these cyanobacterium could therefore be used to transform crop species thereby conferring herbicide resistance.

These cyanobacterial mutant genes, isolated from cyanobacterial sources, can be useful not only for herbicide resistance but also as selectable markers for herbicide, fungicide and insecticide resistance genes as well as output trait genes, as a component for a selection systems when coupled with the imidazolinones and other herbicides. Such a selectable marker



system for nuclear or plastidic transformation could be used for major monocot and dicot crops such as maize, wheat, barley, canola, rice, tobacco, and soybean.

Thus, the present invention provides for improvements to current methods for identifying and utilizing cyanobacterial genes for modifying plant traits including herbicide resistance. Improvements include methods for screening of compounds to identify novel herbicidal modes of action and identify novel herbicide resistance mutations.

Thus, in preferred embodiments, the present invention provides a nucleic acid fragment encoding a herbicide resistant acetolactate synthase (*ahas*) large subunit gene from the cyanobacterium *Synechocystis* PCC 6803 and was cloned from a genomic DNA library.

In further preferred embodiments, a nucleic acid fragment encoding a herbicide resistant acetolactate synthase (*ahas*) small subunit gene from the cyanobacterium *Synechocystis* PCC 6803 was cloned from a genomic DNA library.

In additional preferred embodiments, this invention provides cyanobacteria as an alternative source of *ahas* and *pds* genes for plant transformations, in particular genes encoding herbicide insensitive proteins, and elements of genes for control of expression in plastids.

The present invention also provides a method for the improved genetic transformation of *Synechocystis*.

Finally the present invention provides for the use of the cyanobacterial *pds* and *ahas* genes as a selectable marker for transformations, and as a means of selection with the imidazolinones and 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide for herbicide resistance.

Additionally provided for is a method for target site gene identification, specifically, protocols for "High-Through-Put" molecular manipulation of cyanobacteria *Synechocystis*.

This High-Through-Put system allows us to determine the mode of action of commercial and/or novel compounds for which the mode of action is unknown.

The integration of the processes, along with the described improvements, of;

screening for *Synechocystis* mutants resistant to herbicides, the preparation of genomic DNA from the mutants, the transformation of *Synechocystis* of fragments of mutant DNA, and the identification of transformants which are conferred herbicide resistance from the DNA fragments, and the sequencing of the DNA fragment to identify the target of the herbicide, the mutation conferring herbicide resistance, combined provide for an improved method for identifying novel Mode of Action and mutations within genes for altered traits.

## BRIEF DESCRIPTION OF THE FIGURES/DRAWINGS

### Figure Legends

- Figure 1.** Selection of true resistant mutants.
- Figure 2.** Identification of *Synechocystis* resistance mutant. (Resistant colonies plated on 5µM 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide plates).
- Figure 3.** Susceptibility test of wild type and 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide, mutants of *Synechocystis* using the paper disc assay. (Inhibition of wild-type *Synechocystis* in paper disc assay).
- Figure 4.** Dose-response curve of *Synechocystis* wild type and 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistant mutants after seven days of suspension culture.
- Figure 5.** HighThroughPut (HTP) Target Site Gene Identification in *Synechocystis*.
- Figure 6.** In vivo growth of *Synechocystis* PCC 7120 and *Anabaena* PCC7120 cultured in BG-11 media in the presence of increasing concentrations of PURSUIT® imazethapyr and OUST® sulfometuron methyl.
- Figure 7.** In vitro activity of *Synechocystis* PCC 7120 and *Anabaena* PCC7120 AHAS with increasing concentrations of PURSUIT® imazethapyr and OUST® sulfometuron methyl.

- Figure 8.** PURSUIT® imazethapyr Spray Test Results
- Figure 9.** Amplification of aadA and snAHAS Fragments
- Figure 10.** AHAS Enzyme Assays
- Figure 11.** p12deltaNI construct, also known as pACBC 111
- 5 **Figure 12.** p12deltaNII construct, also known as pACBC112
- Figure 13.** p116I construct
- Figure 14.** p116II construct
- Figure 15.** List of Constructs, including p116

## 10 DETAILED DESCRIPTION

### A. Rapid plate based assay for identifying lead compounds

A prerequisite to successful utilization of *Synechocystis* in target site gene discovery is the identification of compounds that affect the metabolism of this organism. To this end, we have developed and established a rapid plate based assay for screening compounds

15 inhibiting phytoene desaturase (pds) activity. In preferred embodiments the present invention provides improvements in the methods utilizing cyanobacteria, a paper disc assay and a microtitre liquid test, for the screening of novel herbicidal modes of action and to identify novel herbicide resistance mutations. Screening can be performed in simple media, preferably BG-11 (Sigma, St. Louis Missouri), without the need to maintain axenic

20 conditions. Furthermore, quantitative determinations can be made within one to three days.

Screens can be designed to identify inhibitors of other specific metabolic pathways, which are common only to photoautotrophic cyanobacteria and higher plants and not heterotrophic organisms such as other bacteria.

To identify a target site gene activity, two types of Bluegreen algae, *Synechocystis*

25 PCC 6803 (American Type Culture Collection, Rockville, Maryland) and *Anabaena* PCC 7120 (American Type Culture Collection, Rockville, Maryland) can be used for the screen.

One is grown in microtiter dishes in BG-11 medium supplemented with various concentrations of the test compounds. Inhibition of growth can be monitored by visual inspection after two to three days of culture. Quantitative growth measurements can be taken photometrically starting one day after inoculation.

5 Alternatively screens can be performed on agar plates with “lawns” of cyanobacteria and paper discs impregnated with test compounds. In this case, zones of inhibited growth around paper discs can be detected after two or three days.

To set up the assay, wild type cells of *Synechocystis* were mixed with equal volumes of 2x top agar and 2x BG-11 and overlaid on top of BG-11 agar plate. Cells normally appear  
10 in 3-5 days after plating. This method will yield an even and uniform lawn of cells. Upon solidifying, test compounds are then spotted on Whatman filter paper disc before being placed on agar plates. Four compounds can be tested on a single plate. Using this screening, in an example employing 160 different compounds, predominantly compounds of novel mode of action, have been tested on this microbe, and on average, 25% of the compounds show at least  
15 some activity.

### Example 1: Cyanobacterial Screening Process

Rapid plate based assay for screening lead compounds was developed as follows.

20 First, either one of two types of bluegreen algae, *Synechocystis* PCC 6803 and *Anabaena* PC 7120 were grown in microtitre dishes containing BG-11 supplemented with various concentrations of 160 different test compounds. Alternatively, screens can be performed on agar plates with lawns of cyanobacteria and paper discs impregnated with test compounds.

Susceptibility of *Synechocystis* to 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide was tested using a paper disc assay in which 4' fluoro-6-  
25 [(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide was spotted in a paper disc before

being placed on a lawn of cells. In determining susceptibility, the size of the zone of inhibition is indicative of the potency of the compound.

These experiments also established a dose-response curve. A lethal concentration for resistant mutant selection was 1-2 $\mu$ M of 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide. Dose-response studies were also performed in 96-well microtiter plates on wild type and putative 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistant mutants of *Synechocystis*. The growth of the culture was measured daily at an optical density of 690 nm.

A rapid plate based assay for screening and identifying active compounds is then performed. Wild type cells of *Synechocystis* are mixed with equal volumes of 2x top agar and 2x BG-11. The mixture is then overlaid on top of a BG-11 agar plate. Cells normally appear 3-5 days after plating. This method will yield an even and uniform lawn of cells.

After solidification of the agar, test compounds were spotted on Whatman filter paper discs, and then were placed on agar plates. Four different compounds were tested on a single plate. Using this screening method, 160 different compounds were tested, predominantly compounds of novel mode of action. On average, 25% of the compounds show at least some activity.

## **B. *Synechocystis* mutant pds gene**

The protein phytoene desaturase (PDS, encoded by the gene *pds*) is the target of a number of commercially valuable bleaching herbicides. The simple cyanobacterial genetic system, *Synechocystis*, was used to generate and select mutant forms of *pds* resistant to 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide. (BASF (Previously Cyanamid), Princeton, New Jersey)

4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide is a herbicide for the post-emergence control of broad leaf weeds in winter and spring wheat. Its site of action has been determined to be PDS. On BG-11 (Sigma, St. Louis Missouri) solid medium in a

paper disc assay, 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide was found to be active against *Synechocystis* PCC 6803 (Referred to as *Synechocystis*) at concentrations in the 1-2  $\mu$ M range. Furthermore, *Synechocystis* growth was inhibited with an  $I_{50}$  in the lower sub-micromolar range when it was tested in liquid cultures.

Thus, the present invention provides novel *Synechocystis* mutant phytoene desaturase (*pds*) gene(s) conferring resistance to 4'-fluoro-6-[(alpha, alpha, alpha,-trifluoro-m-tolyl)oxy]-picolinamide.

The present invention provides a method to isolate and select mutants resistant to 4'-fluoro-6-[(alpha, alpha, alpha,-trifluoro-m-tolyl)oxy]-picolinamide. Two types of mutants may be isolated, spontaneously produced mutants or chemically induced mutants. Chemically induced mutations may be induced with, for example, ethyl methanesulfonate (EMS).

Spontaneous mutants were obtained by growing wild-type *Synechocystis* in liquid culture, or directly plated on plates containing lethal concentrations of 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide or through stepwise exposure to increasing levels of 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide in liquid culture. Putative resistant colonies were then plated on selection plates to obtain single resistant cell lines.

For isolating EMS induced mutants, *Synechocystis* cell cultures were treated with EMS at a concentration which gives 99% killing, followed by growth on selection plates. ~200 ml samples of logarithmic liquid culture were harvested and treated with EMS. The reaction was stopped by addition of sodium thiosulfate, to a final concentration of 5%, to quench excessive EMS. Cells were then collected and washed twice with BG-11. After an overnight recovery in fresh BG-11 medium, cells were plated on solid BG-11 medium containing 1  $\mu$ M 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide.

To select the 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistant mutants, surviving colonies of the EMS treatment were picked and cultured in BG-11 in 96-well microtiter plates. After 2-4 days growth, cells were replica plated on BG-11

plates containing 0, 2, or 5  $\mu\text{M}$  4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide to identify true resistant mutants. **Figures 1A, 1B and 1C** show the results from one set of selection plates. As the concentration of 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide was increased from 2 to 5  $\mu\text{M}$  (from Figure 1B to 1C), the majority of the cells fail to grow. Out of 576 (96x6) putative resistant colonies plated on 5  $\mu\text{M}$  of 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide plates, 7 resistant colonies were identified, as shown in **Figures 2A, 2B, 2C and 2D**.

The resistance phenotype of selected mutant cell lines was further tested in solid medium as well as in suspension cultures. Selected resistant colonies were given in-house names to differentiate themselves from one another: 5-1/12E, 5-1/12F, 7-2/1E, 7-3/11F, 7-3/12F, 7-4/12F. **Figures 3a, 3b, 3c, 3d, 3e, 3f and 3g** shows the growth of wild type *Synechocystis* was significantly inhibited at a rate of 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide as low as 0.5 nmol whereas the growth of mutant lines was inhibited at a substantially lesser rate. The difference between the wild type and mutant lines becomes even more apparent at the highest rate (5 nmol) tested.

In this particular experiment and for the wild type *Synechocystis* cells, zones of inhibition were observed at the two higher 4'fluoro-6-[(alpha, alpha, alpha,-trifluoro-m-tolyl)oxy]-picolinamide application rates ( $5 \times 10^{-10}$  mol and  $5 \times 10^{-9}$  mol) with a diameter of 20 and 38 mm, respectively. However, zones of inhibition were only observed with 4 of the 6 mutants at the highest rate of 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide, results with degree of resistance in the following order: 7-3/11F(0) = 7-4/12F(0) > 5-1/12E(8) > 7-3/12F(12) > 5-1/12F(18) > WT(38) (size of zone in mm in parentheses)

In suspension cultures, all mutants exhibit increased resistance against 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide. **Figure 4** shows the result from one such dose-response experiment after seven days of culture. For wild type cells (WT), the growth was inhibited at concentrations of 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-

tolyl)oxy]-picolinamide of  $<0.25\ \mu\text{M}$ , with an  $I_{50}$  in the sub  $\mu\text{M}$  range. By contrast, the  $I_{50}$  values are between 1-2  $\mu\text{M}$  for 5-1/12E, 5-1/12F, and in the range of 5-10  $\mu\text{M}$  for 7-3/12F and 7-4/12F, respectively.

Thus, there is substantial evidence that the isolated cell lines confer increased levels of 4' fluoro-6-[(alpha, alpha, alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistance.

## Example 2: Isolation and Selection of Mutant PDS Genes

100-200 ml of logarithmic liquid culture was harvested and treated with mutagen ethyl methanesulfonate (EMS) in a phosphate buffer. To quench excessive EMS, the reaction was stopped with the addition of sodium thiosulfate to a final concentration of 5%. Cells were collected and washed twice with BG-11, then placed in a fresh BG-11 medium for overnight recovery.

The cells were then plated on a solid BG-11 medium containing 1  $\mu\text{M}$  4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide. Surviving colonies were cultured in BG-11 within 96-well microtiter plates.

To identify true mutants, cells were replica plated on BG-11 plates containing 0, 2 or 5  $\mu\text{M}$  4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide, after 2-4 days growth.

A result from one set of figure plates is shown in Figures 1A, 1B and 1C. As the concentration of 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide was increased from 2 to 5  $\mu\text{M}$ , the majority of the cells failed to grow. Only 7 resistant colonies were identified out of the 576 (96x6) putative resistance colonies plated on 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide. Figures 2A, 2B, 2C and 2D.

The resistant phenotype of selected mutant cell lines was further tested in solid medium as well as in suspension cultures. For the solid medium tests, a paper disk assay was done. As shown in Figure 3, the growth of wild type *Synechocystis* was significantly inhibited



with 0.5 nmol of 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide. In contrast, the growth of the mutant lines was inhibited at a lesser rate. The difference between the wild type cells and the mutant cell lines become more apparent with a higher concentration of 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide.

5 In a suspension culture test, all mutants exhibited increased resistance against 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide. **Figure 4** shows the result from one such dose response experiment after seven days. Wild type cells were inhibited at concentrations of <0.25  $\mu$ M 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide, with an  $I_{50}$  in the sub  $\mu$ M range. In contrast  $I_{50}$  values are between 1-2  $\mu$ M for 5-1/12E and 5-1/12F, and between 5-10  $\mu$ M for 7-3/12F and 5-4/12F. Thus, because the cell lines with the mutant *pds* genes are far more resistant than wild type cell lines, there is evidence that the selected cultures contain 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistance.

15 A method for the preparation of *pds* resistant nucleic acid fragments from the cyanobacteria *Synechocystis* EMS resistant cell lines is provided in additional preferred embodiments.

Genomic DNA was prepared from six *Synechocystis* EMS resistant cell lines obtained from the isolation and selection process above. A 1.7 Kb Genomic DNA fragment encompassing the *pds* was amplified using Genomic DNA as a template. PCR amplified *pds* gene fragments were subsequently subcloned into the Invitrogen TOPO TA Cloning vector pCR2.1-TOPO (Invitrogen Corp, Carlsbad, California) to obtain plasmid pCR2.1-TOPO-PDS.

25 Cloning of the resistant *pds* gene into a vector was done as follows. A pair of primers were designed based on sequence information available in a database (database available at [www.ncbi.nlm.nih.gov/cgi-bin/Entrez/eframik?db=Genome&gi=112](http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/eframik?db=Genome&gi=112) or [www.kazusa.or.jp/cyano/kwd.html](http://www.kazusa.or.jp/cyano/kwd.html)). The primers had the sequence (from 5' to 3'): X62574-5' cgaattccctgtagcatttaatacaattggc , identified as Sequence ID NO: 1 and X62574-3'

cgcataagctttgcagatggagacggtttgggc, identified as SEQ ID NO: 2. The primers were used to amplify the *pds* gene encoding phytoene desaturase, using *Synechocystis* Genomic DNA (prepared from six *Synechocystis* EMS resistant cell lines obtained from the isolation and selection process above) as a template. A 1.7 Kb PCR fragment was obtained and subsequently subcloned into Invitrogen TOPO TA vector to generate plasmids TOPO TA-PDS (PDSr).

### Example 3: Cloning and Subcloning of Mutant PDS Gene

Cloning of the mutant *pds* genes went as follows. A pair of primers were designed to amplify the *pds* gene using *Synechocystis* DNA prepared from wild type and 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistant mutant cells as templates.

PDS genes were cloned from wild type *Synechocystis* and 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistant cell lines. *Synechocystis* genes were cloned from cell lines by a PCR based strategy. Genomic DNA was used as a template. Based on sequence information available in a database, the following primers were used (from 5' to 3'): X62574-5' cgaattccctggtagcatttaatacaattggc, SEQ ID NO: 1, and X62574-3' cgcataagctttgcagatggagacggtttgggc, SEQ ID NO: 2.

A 1.7 Kb PCR fragment was obtained and subsequently subcloned into Invitrogen TOPO TA vector, resulting in plasmids TOPO TA-PDS (PDSr). PCR products were subcloned into an Invitrogen TOPO TA cloning vector, generating TOPO TA-PDS (PDSr). Plasmids carrying *pds* insertion were prepared using Qiaprep Spin Miniprep Kit. (Qiagen Inc., Valencia, California).

PDS gene PCR products as well as plasmids carrying *pds* gene derived from all six mutant cell lines were used in a functional complementation assay.

Testing was done to eliminate the possibility that 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistance was linked to a mutation other than, or in

addition to, the phytoene desaturase in *Synechocystis*. Digested *Synechocystis* genome DNA, PCR fragments of PDS gene and TOPO TA-PDSr plasmids were all used in a genetic complementation study. All DNA species tested transformed *Synechocystis* to 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistance. This suggests that resistance to 4'fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide is associated with the mutation in the *pds* gene in these mutant cell lines.

Three independent clones were picked and sequenced for each mutant cell line. Sequencing of PCR amplified *pds* gene product from resistant cell line 7-4/12F revealed a single base pair change of G⇒A at position 642 (position 523 within ORF) (**Table 1**), resulting in an amino acid change of Ala⇒Thr at position 175. The sequence is identified as Sequence ID NO: 3. This mutation is unique and different from the only mutation (Arg<sup>195</sup>⇒Cys, Pro, or Ser) described in the *pds* gene from *Synechocystis* by [Sandmann et al., 1998], and four other point mutations (Arg<sup>195</sup>⇒Pro, Leu<sup>320</sup>⇒Pro, Val<sup>403</sup>⇒Gly, Leu<sup>436</sup>⇒Arg) previously reported for the *pds* gene from *Synechococcus* sp. PCC 7942. All of the previously described mutations were identified based on their ability to confer resistance to the commercial herbicide norflurazon to wild type cells.

**Table 1.** List of point mutations in herbicide resistance-conferring *pds* genes from cyanobacteria

Amino Acid Position	Mutation	A.A.Substitution	Source	Target Herbicide	References
175	G⇒A	Ala⇒Thr	<i>Synechocystis</i>	4'-fluoro-6-[(alpha,alpha,alpha-trifluoro-m-tolyl)oxy]-picolinamide	This work
195	C⇒T	Arg⇒Cys	<i>Synechocystis</i>	norflurazon	Martínez-Férez & Vioque 1992
195	C⇒A C⇒T G⇒C	Arg⇒Ser Arg⇒Cys Arg⇒Pro	<i>Synechocystis</i>	norflurazon	Martínez-Férez <i>et al</i> 1994
403	T⇒G	Val⇒G	<i>Synechococcus</i>	norflurazon	Chamovitz <i>et al</i> 1991
195 320 436	G⇒C T⇒C T⇒C	<u>Arg⇒Pro</u> <u>Leu⇒Pro</u> Leu⇒Arg	<i>Synechococcus</i>	norflurazon	Chamovitz <i>et al</i> 1993

#### 5 Example 4: Sequencing of Mutant PDS Gene

Three independent clones were picked and sequenced using the dRhodamine Terminator Cycle Sequencing Kit. (PE Biosystems, Norwalk, Connecticut). The reactions were analyzed in an ABI A310 Genetic Analyzer (ABI, Foster City, CA.) Sequencing the PCR amplified *pds* gene product from resistance cell line 7-4/12F revealed a single base pair change of G⇒ A at position 642 (position 523 within ORF) See Table 1. The result is an amino acid change of Ala ⇒ Thr at position 175. The mutation is unique. It is different from the only mutation described in the *pds* gene from *Synechocystis* (ARG195 ⇒ Cys, Pro or Ser), and four other point mutations previously reported for the *pds* gene from *Synechocystis* sp. PCC 7942 (Arg195 ⇒ Pro, Leu320 ⇒ Pro, Val403 ⇒ Gly, Leu436 ⇒ Arg). All of those mutations were identified based on their ability to resist commercial herbicide norflurazon to wild type cells.

The complete sequence of the novel mutant form pds gene, identified as SEQ ID NO:

3, reads as follows:

1 **ccctggtagc atttaataca aattggctat** ctggcacaag tccccgaaa tattacgaaa  
5 61 cgtaaagtat aataacaatc aacctgtaaa ccccaaatgc cftagcgaga cagtaaccca  
121 tgcgcgttgt gatcgccgga gccggattag ccggcctagc ctgtgccaaa tacttagccg  
181 atgcgggctt tacccccgtc gtcttgaac gtagggatgt attaggcggg aagatcgccg  
241 cgtggaaaga tgaggacgga gattgttacg aaaccggcct acacatttt ttggggcct  
301 atccaacat gttgcagtta tttaaggaat tggatatcga agatcgtctg caatggaaa  
10 361 agcacagcat gatctcaac caaccagaga aaccaggtag ctactctcg ttcgatttc  
421 cggatattcc ggccccatc aatggttg tagccattct tcgcaacaac gatatgcta  
481 cctggccgga gaaaattcg ttggcttg gactctgcc ggccattgc cagggccaga  
541 gctatgtga agaaatggat aaatacacti ggtcagagt gatggccaaa caaaatattc  
601 ccccccat cgaaaaagaa gtttcatf ccatgagtaa gacgttgaac ttattgatc  
15 661 ccgatgaaat ttccgccacc atttactta ctgccctcaa tcgctttta caggaaaaa  
721 atggctctaa gatggcatc ctggatgggg caccaccgga gcgtcttgc caaccttgg  
781 tcgactatat tacggaacgg ggagggaag tacacaffaa taaacctctc aaagaaatt  
841 tgctaatga agatggttcc gtaagggtt acttaatccg gggcctagat ggagccccg  
901 acgaagtgat cacagcggat ttatatgtgt ctgcatgcc ggtggatccc ctgaaaacca  
20 961 tgggtccagc gccctggaga gaatacctg agtttaagca aatccaaggt ttggaaggag  
1021 tcccggtcat taacctccac ctgtggttg accgtaagti aaccgacatt gatcattgt  
1081 tatttcccc atcgccgttg ttgagtgtt acgccgacat gagcaacacc tgccgagaat  
1141 acagtgatcc agacaaatcc atgttgaat tgggtctggc tccggcccag gattggatc  
1201 gcaaatccga cgaagagatt gtggcggcca ccatggcgga gatcaagcaa ctcttcccc  
25 1261 aacactcaa cggggataat ccagcccga tgcttaaat ccacgtgtc aaaaccccc  
1321 gctcagtcta caaagctacc cccggaaggc aggctgtcg cccgatcaa cggacatcg  
1381 tgcccaactt ttacctagca ggggacttca ccatgcaaaa atactgggc agtatggaag

1441 gggcgggtgct ttccggcaaa caatgcgccc aggcgatgc cgccgatttc aacccccaaa  
 1501 ccgttccccc caccagggaa atagtcaccg tgggtaagc cgcttgact ccctggtaat  
 1561 ctctctgaca aatggcaacc ctaatgcgac aatgctaaat ggctaacggt caaatttctc  
 1621 cccagcgtgc agttacaaaa cccaatcct ggtggctgac ttccgaaccc cgtccgtcct  
 5 1681 taatgttaca **actgccaaa cgtctccat ctgcaaagcc** ctgtgcttct gttga

The 5' PCR primer with an engineered EcoR I (Promega) site was highlighted in bold, and that of the 3' PCR primer with an engineered Hind III (Promega) site was also bold typed. The novel substitution of G → A at position 642 (position 523 within the PDS ORF) is boxed.

In further embodiments we provide a method for the improved genetic transformation of *Synechocystis*. In the literature, transformation of *Synechocystis* has been performed using either one of the two approaches, "in situ" dot transformation first reported by Dzelzkalns & Bogorad (*The EMBO J.*, 1998, 7: 333-338), and liquid culture based transformation (ref. Williams, *Methods in Enzymology* 1988, 167: 766-778). For the liquid culture based procedure, DNA samples were mixed with fresh cells of *Synechocystis* and incubated for certain period of time before being spread onto membrane filters resting on BG-11 agar plates. After an extended incubation of the plates under standard conditions for the expression of inserted gene(s), the filters were transferred to plates containing selection agents. This is a lengthy procedure and may not be suitable for High-Through-Put transformation.

The "in situ" dot transformation procedure entails direct application of DNA sample (restriction fragments, cloned plasmids) in liquid or melted agarose onto a lawn of *Synechocystis* 6803 cells containing selection agents. It is quick and convenient, but cells were not given the time to express the inserted gene before being exposed to selection agents, this procedure is also "destructive" in that DNA samples will be lost regardless of transformation results.

*Synechocystis* DAN was prepared using the Qiagen Dneasy Plabti Mini Kit (Qiagen, Valencia, California) following NaI pretreatment and digestion with lysozyme as describes in Williams (1988). For manipulation of DNA in *E. Coli*, standard recombination procedures were followed.

5 A much-improved method was developed in our laboratory to overcome the limitations of the 'in situ' dot transformation and the liquid culture based transformation methods. To transform *Synechocystis*, competent cells were arrayed in 96-well plates. The DNA species to be transformed were then added and mixed with the cells. The 96-well plates containing mixtures of DNA and cells were then placed in a Sumilon plate (Vangard International Inc., Taipei, Taiwan) moistened with wet sterile paper towels. Cells were replica-plated at various times onto selection plates containing various concentration of the same or different selection agents. This method is extremely suitable for performing transformations and screening of a large number of samples, such as with the High-Through-Put protocol in Section C.

10 Transformation of wild-type *Synechocystis* with either DNA species results in enhanced 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]picolinamide resistance. This reinforces the notion that resistance in the original cell lines is the result of mutation within the *pds* gene.

Also provided for in preferred embodiments is 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]picolinamide resistant mutants which show cross-resistance against other PDS inhibitors. These mutants, when tested against another herbicides which are PDS inhibitors, compound (2E)-2-[amino(benzylsulfanyl)methylene]-1-(2,4-dichlorophenyl)-1,3-butanedione and two of its analogs, pyridine, 2-[(3,3-dichloro-2-propenyl)oxy]-4-methyl-6-[[2-(trifluoromethyl)-4-pyrodinyl]oxy] and 1,2,4,5-benzenetetracarboxamide,N,N',N'',N'''-tetrakis[5-(benzoylamino)-9,10-dihydro-9,10-dioxo-1-anthracenyl], exhibited cross-resistance.

### C. High-Through-Put target site gene identification using *Synechocystis*

In this invention, we further describe the successful development of various protocols for High-Through-Put (HTP) molecular manipulation of *Synechocystis*. These include but not limited to procedures such as lead compound identification, generation and selection of resistant mutant, HTP genetic transformation and functional complementation. As a result, it is now possible to design a program for rapid and cost effective identification of target site genes using this microbe.

As illustrated in **Figure 5**, a prerequisite to the successful implementation of this program is the identification and availability of lead chemicals active on this microbe.

Resistant mutants can be generated and selected against the compound of interest using a chemical based approach. To isolate the resistance-conferring gene, one of the most commonly adopted practices has been the gel fractionation method. This method entails the following steps: (1) digestion of genomic DNA prepared from mutant cell cultures of *Synechocystis*, (2) fractionation of digested DNA on agarose gel and purification of DNA from gel slices, (3) identification of positive fraction through 1<sup>st</sup> round of functional complementation, (4) construction of a gene library, (5) preparation of plasmid DNA from single colonies, and (5) identification of target gene in 2<sup>nd</sup> round of functional complementation. This is a very time-consuming process. There is a possibility that the resistance-conferring fragment may not be the right size for complementation assay and/or for subsequent subcloning into library vector. Consequently, the gene fragment of interest may never be found in the gene library. By contrast, a preferred embodiment of the High-Through-Put program requires the preparation of ~1800 primer pairs for amplification of 1800 **overlapping** 2-kb fragments (the size of the fragment, thus the total number of primers, may be altered for easy PCR amplification and HTP manipulation) to cover the complete genome of *Synechocystis* (~3.6 Mb). It entails rapid amplification of 1800 fragments using genomic DNA from *Synechocystis* mutant cell lines.



A size range of 1.5~3 kb would be ideal, both for PCR amplification and homologous recombination in *Synechocystis*. PCR products that are too small would compromise the efficiency of transformation in this microbe. On the other hand, it is more difficult to amplify bigger gene fragment using PCR. Some trial and error adjustment can be made as needed in a particular PCR system according to methods well known to those skilled in the art. This process can be adapted to any organisms (e.g. Yeast *S. cerevisiae* or other cyanobacteria) for which the whole genome sequence information is known and transformation through homologous recombination is feasible.

PCR products can then be used for HTP transformation of *Synechocystis* and functional complementation assay on various selection plates, using methods well-known to those skilled in the art. Gene(s) conferring herbicide resistance can then be identified based on the ability of its PCR products to confer herbicide resistance to wild type cells upon transformation. All of which can be performed using 96-well microtitre plates, in addition, only one round of transformation is needed to identify the resistance-conferring gene. Some major steps in this process are detailed below:

(1) **Lead compounds identification:** This can be done in a reasonably high throughput manner using either the paper disc assay on solid BG-11 agar plate or 96-well microtiter plate as described in Section A and Examples 3 & Example 4.

(2) **Generation and isolation of resistant mutant(s):** *Synechocystis* mutant(s) resistant to compound of interest can be generated chemically by treating cultures of *Synechocystis* with chemical mutagens (e.g. EMS). Procedures for performing such experiment are provided in Examples 6 & 10.

(3) **Isolation of genomic DNA from resistant cell lines:** Genomic DNA can be prepared from cultures of *Synechocystis* resistance cell lines using commercial kits (e.g. Qiagen DNAeasy Plant Kit) as described in Section B and Example 7.

**(4) Primer design and PCR amplification of gene fragments from *Synechocystis*:**

Primer pairs for amplification of overlapping DNA fragments from *Synechocystis* can be designed with the assistance of a commercial software package (e.g. Vector NTI from InforMax, North Bethesda, MD). Large-scale synthesis of primers can be done by a commercial vendor (e.g. Sigma-Genosys, The Woodlands, TX) in 96-well format. PCR amplification of ~1800 2-kb fragments (*again, the size of the fragment, thus the total number of primers may be altered for easy PCR amplification and HTP manipulation*) can be performed using genomic DNA prepared from mutant cell cultures as template following standard laboratory procedures (Example 9).

**(5) High Through Put genetic transformation and target site gene identification:**

Procedures for HTP genetic transformation and functional complementation assays have been described in Section B and Example 8. Gene(s) conferring herbicide resistance can then be identified based on the ability of its PCR products to confer herbicide resistance to wild type cells upon transformation.

This program offers the flexibility of working with more than one active compound at a time. This flexibility occurs because of the ease with which one can replica-plate cells on plates containing different selection compounds, at different time upon transformation with PCR products. Conceivably, this will be a very high through put process for the rapid identification of target site genes once active compounds are identified.

**D. *Synechocystis* AHAS Genes**

AHAS physical properties

Cyanobacteria are a particularly useful source of genes for enhancing crop performance due to their similarity, and ancestral connection, to plant chloroplasts. In particular, cyanobacterial genes may be useful for transformation directly into the chloroplast

genome due to similarities in genetic elements. Similarities in cyanobacterial genes and proteins to those from chloroplasts can carry over to a shared susceptibility to herbicides. *Synechocystis* PCC 6803 was demonstrated to be susceptible to several known herbicides as shown in **Table 2** as described in detail below.

5

**Table 2.**

Compound	Activity Rating (++ = highest)	Target Site
Maleic Hydrazide	++	Carotenoid Biosynthesis
Simazine	++	Photosynthesis
Fenuron	++	?
Monuron	++	Photosynthesis
CMU	+	Photosynthesis
Desmedipham	++	?
Bromoxynil	+	Photosynthesis/ Respiration
Phenmedipham	++	?

10

In cases where cyanobacteria are susceptible, they are good organism for use in screening for mutations that confer resistance due to the readily available methods for genetic manipulation such as transformation, high throughput screening, liquid or agar based selection, replica plating, shuttle vectors, a small, and in some cases a completely sequenced, genome. The mutated gene sequences that are isolated after selection for resistance can be transformed into the nucleus or plastome of plants, or alternatively, the functional equivalent of identified mutations can be inserted into genes from plants or other organisms for use in transformations.

15

In some cases cyanobacteria are insensitive to herbicides, potentially due to differences in uptake, metabolism, or differences in the target protein. Consequently, genes from cyanobacteria may be useful in conferring herbicide resistance to plants of interest.

20

### AHAS biochemistry

The end products of the branched chain amino acid biosynthetic pathway (isoleucine, leucine, and valine) feedback inhibit Acetohydroxyacid synthase (AHAS) activity. Only the large subunit has catalytic activity.

5 It has been established in the literature for many years that microbial AHAS enzymes, *in-vivo*, exist as two distinct but physically associated protein subunits. The two polypeptides, a "large subunit" and a "small subunit" are expressed from separate genes. From the study of AHAS enzymes from microbial systems, two roles have been described for the small subunit: 1) the small subunit is involved in the allosteric feedback inhibition of the  
10 catalytic large subunit when in the presence of isoleucine, leucine or valine and, 2) the small subunit enhances the activity of the large subunit in the absence of isoleucine, leucine or valine. For example, the large subunit alone has a basal level of activity that cannot be feedback inhibited by amino acids. When the small subunit is added, the level of activity of the large subunit increases. If the small subunit is included with isoleucine, leucine or valine,  
15 the activity is below that of the basal level with large subunit alone.

Since activity of prokaryotic AHAS large subunits have been shown to be suboptimal in the absence of small subunits, the level of activity of the *Synechocystis* AHAS large subunit, and its ability to confer herbicide resistance, may be suboptimal without co-expression of a small subunit gene.

20 The sequence of the entire genome of the cyanobacterium *Synechocystis* PCC 6803 has been determined and published.

(<http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/fragik?db=Genome&gi=112> or

<http://www.kazusa.or.jp/cyano/kwd.html>) When the genome of *Synechocystis* PCC 6803 was published, subsequent to cloning of the original AHAS large subunit gene a search was done  
25 on the genome for other AHAS genes. The search found an additional gene with a high degree of homology to AHAS sequences. This gene in *Synechocystis* is designated sl11981 and annotated as *ilvB*.

However, prior to the publication of *ilvB* sequence, we cloned a novel *Synechocystis* *AHAS* Large Subunit Gene nucleic acid fragment cloned from a genomic DNA library of cyanobacterium *Synechocystis* PCC 6803. This original gene that was cloned is identified as slr2088 and annotated as *ilvG*. Susceptibility tests show that AHAS activity is resistant to imidazolinones such as PURSUIT® imazethapyr (BASF, formerly American Cyanamid, Princeton, New Jersey) and sulfonylureas such as OUST® sulfometuron methyl (DuPont, Wilmington, Delaware).

In vivo resistance of cyanobacteria to PURSUIT® imazethapyr and OUST® sulfometuron

methyl As a preliminary matter, *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 were tested for susceptibility to PURSUIT® imazethapyr and OUST® sulfometuron methyl. AHAS genes which are resistant to these herbicides are excellent candidates for transformation in plant plastomes and nuclear genomes. Such transformants can be used in a weed control strategy using a combination of transgenic herbicide resistant crops and herbicides.

In vivo testing of *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 was done by culturing the organisms in varying concentrations of the commercial herbicides. Both organisms demonstrated a high degree of insensitivity to the compounds (**Figure 6**). No inhibition of growth was seen at concentrations of 100 µM PURSUIT® imazethapyr or 100 nM OUST® sulfometuron methyl after one week of culture in BG-11 media. For relative comparison purposes a concentration of 1 µM PURSUIT® imazethapyr in agar media is lethal to *Arabidopsis* plants.

In vitro resistance of cyanobacterial acetohydroxyacid synthase to PURSUIT® imazethapyr and OUST® sulfometuron methyl

AHAS is the target site of both PURSUIT® imazethapyr and OUST® sulfometuron methyl herbicides. To determine if resistance to the herbicides is due to a natural resistance to inhibition of the acetohydroxyacid synthase enzymes from the cyanobacteria, or if it is due to alternative mechanisms (e.g. lack of entry into the cell), the *in vitro* activity of the AHAS enzyme in the presence of the herbicides was tested.

AHAS assays were performed with slight modification as described by Singh *et. al* (Singh BK, Stidham MA Shaner DL, 1988, Assay of acetohydroxyacid synthase from plants. Anal Biochem 171: 173-179).

Results from the *in vitro* assays (**Figure 7**) demonstrates that both *Synechocystis* and *Anabaena* AHAS enzymes are insensitive to inhibition by the herbicides. The  $I_{50}$  of plant AHAS enzymes are normally in the range of 1-2  $\mu$ M for imidazolinones and 10 nM for sulfonylureas (Singh, B.K., Stidham, M.A., and Shaner, D.L., J. Chromatogr., 444, 251, 1988). No significant inhibition of the cyanobacterial AHAS enzymes was observed at concentrations of 100  $\mu$ M PURSUIT® imazethapyr and 100 nM OUST® sulfometuron methyl.

The data shown in **Figure 7** indicated that resistance to AHAS inhibiting herbicides could be attributed to the natural resistance of the target enzyme. Thus, cyanobacterial AHAS genes would be good candidates for transformation into plants, either by nuclear or plastid transformation, for conferring herbicide resistance.

Also, there is a level of cross-resistance exhibited between PURSUIT® imazethapyr and sulfanylcarboxamides. As discussed below, certain lines transformed with the p116 plasmid constructs (**Figure 9**) described in detail below, when sprayed with 18g/ha PURSUIT® imazethapyr showed about a 20% increase in plant resistance in the presence of PURSUIT® imazethapyr and sulfanylcarboxamides when compared with wild type tobacco. Interestingly, it appears that the *Synechocystis* AHAS enzyme displays a level of

cross resistance to both PURSUIT® imazethapyr and sulfanylcarboxamides, although the herbicides are both quite dissimilar structurally.

**Example 5: In vitro resistance resistance of cyanobacterial acetohydroxyacid synthase to PURSUIT® imazethapyr and OUST ® sulfometuron methyl .**

Experiments were done to determine in vitro resistance of cyanobacterial acetohydroxyacid synthase to PURSUIT® imazethapyr and OUST ® sulfometuron methyl.

*Synechocystis* PCC 6803 and *Anabaena* PCC 7120 were cultured in 1.5 L of BG-11 media. Cells were collected by centrifuge and stored frozen at -80°C. Frozen cells were thawed and placed in a 30 mL Bead Beater cell disruptor chamber (BioSpec Corp Bartlesville, OK). Seven mL of acid washed sand was added. The chamber was filled with 2X AHAS assay buffer, consisting of 100mM HEPES pH 7.0, 200 mM pyruvate, 20 mM MgCl<sub>2</sub>, 2 mM TPP (Thiamine pyrophosphate) and 100 uM FAD (flavin adenine dinucleotide).

The Bead Beater cell disruptor was packed in ice and turned on for 10 seconds, followed by cooling for 3 minutes. This cycles was repeated five times. The extract was transferred into a centrifuge tube, and spun in a Beckman SA20 (Beckman, Fullerton, California) rotor for 15 minutes at 17,000 rpms.

The supernatant was decanted and used for AHAS assays. The assays were performed with slight modification as described by Singh et. al (Singh BK, Stidham MA Shaner DL, 1988, Assay of acetohydroxyacid synthase from plants. Biochem 171: 173:179). AHAS activity was assayed in a final concentration of 1X AHAS buffer, except HEPES was used instead of phosphate buffer that Singh used. All assays containing PURSUIT® imazethapyr , OUST ®sulfometuron methyl or associated controls contained a final concentration of 5% DMSO (Dimethyl Sulfoxide) due to addition of the herbicides to the assay mixtures as a 50% DMSO stock. Assays were performed in a final volume of 250uL at 37°C in microtiter plates for one hour.

### Isolation of the *Synechocystis* AHAS Large Subunit Gene

The sequence of the coding and flanking regions of the isolated cyanobacterial  
AHAS gene of the present invention, which confers resistance to PURSUIT® imazethapyr  
AND OUST ® sulfometuron methyl, was determined.

A probe for identifying the *Synechocystis* AHAS gene was generated by PCR with  
degenerate primers. To develop these degenerate primers, alignments were made of known  
AHAS sequences from plants, bacteria, and other cyanobacteria, such as *Spirulina platensis*  
(M75907.Gb\_BA and M75906.Gb\_BA, (GenBank, National Center for Biotechnology  
Information). It was found that the predicted amino acid sequences of AHAS protein shared  
many conserved regions. Thus primers were chosen in regions where amino acid sequences  
were highly conserved. Degenerate primers were used to allow for differences in the  
cyanobacterial codon usage. One of the primer pairs, identified as SEQ ID NO:4 and SEQ ID  
NO: 5, respectively had a sequence of:

(SEQ ID NO. 4)

#21: 5' GG(AGCT)AC(AGCT)GA(TC)GC(GACT)TT(TC)CA(AG)GA 3'

(SEQ ID NO. 5)

#19: 5' (CT)T(CG)CCA(CT)TG(AGCT)C(TG)(AGCT)ACCAT 3'

Genomic DNA was isolated from *Synechocystis* PCC 6803 (ATTC # 27150) according  
to Methods in Enzymology 167, p 703-712 and was a template for PCR amplification of an  
AHAS fragment. The 1 kb PCR product corresponded in size to the fragment these primers  
would produce based on the distance between the two conserved regions from which the  
primers were designed. The fragment was isolated and cloned into the pCRII vector  
(Invitrogen). The insert was partially sequenced and the sequence was found to have strong  
homology to both of the



*Spirulina* AHAS sequences (about 80% similarity and about 70% identity at the amino acid level between the *Synechocystis* sequence and the sequence from the M75907.Gb\_BA and the *Synechocystis* sequence and the sequence from the M75906.Gb\_BA *Spirulina* .)

## 5 Example 6: Isolation of the *Synechocystis* Large AHAS Gene

A probe for identifying the *Synechocystis* AHAS gene was generated by PCR with degenerate primers. Genomic DNA was isolated from *Synechocystis* PCC 6803 according to the method outlayed in Methods in Enzymology 167, p/ 703-712. PCR was performed with DNA polymerase (Perkin Elmer AmpliTaq, Perkin Elmer, Shelton, Connecticut) using this genomic DNA as the template and a series of degenerate primers that were designed from the conserved regions observed in the alignment of AHAS gene sequences in Genbank. (www2.ncbi.nlm.nih.gov/genbank/query\_form.html). One of the primer combinations, identified as Sequence ID No.4 and Sequence ID No 5., respectively,:

#21: 5' GG(AGCT)AC(AGCT)GA(TC)GC(GACT)TT(TC)CA(AG)GA 3'

#19: 5' (CT)T(CG)CCA(CT)TG(AGCT)C(TG)(AGCT)ACCAT 3'

produced a 1.1 kb PCR product that corresponded in size to the fragment these fragments would produce, based in the sequences of the two AHAS genes from the cyanobacterium *Spirulina platensis*. The fragment was isolated and cloned in a pCRII vector (Invitrogen) The insert was amplified and partially sequenced, and was found to have strong homology to both of the *Spirulina* AHAS sequences, about 80% similarity, 70% identity at the amino acid level.

### Library Screening

A genomic library from *Synechocystis* PCC 6803 in the Lambda ZAP vector (Stratagene, La Jolla, California) was screened for the AHAS gene. To obtain the probe for screening the *Synechocystis* genomic library, the plasmid isolated in the above procedure was  
5 digested with Eco RI (Promega) and the resulting 1.1 kb fragment was gel isolated and purified (GeneClean, Bio 101, Qbiogene, Carlsbad, California). This material (25-50 ng) was labeled with  $^{32}\text{P}$  following the Oligolabelling Kit Standard Protocol (Pharmacia, Piscataway, New Jersey). Thus labeled, the 1.1 kb fragment was used as a probe to screen for the AHAS gene in the Lambda Zap vector genomic library.

10 The *Synechocystis* PCC 6803 Genomic Library was plated on three plates (NZCYM media) (Sambrook, Fritsch, Maniatis "Molecular Cloning- A Laboratory Manual 2nd Ed" 1989) at a titer of  $5 \times 10^3$  pfu/plate. Duplicate filters (BA-S NC, Schleicher & Schuell) were lifted from each of the plates. The filters were incubated on 15 cm 0.5N NaOH/1.5 M NaCl for 90 seconds, 0.5M Tris/1.5 M NaCl for 5 minutes, and then 2 x SCC (Sodium chloride,  
15 Sodium Citrate, pH 7.0) (Sambrook, Fritsch, Maniatis Molecular Cloning- A Laboratory Manual 2nd Ed. 1989) for 5 minutes.

The filters were then air dried and baked in a vacuum oven at 80°C for two hours. Afterwards, the filters were prehybridized in 50 ml of prehyb solution (50% deionized formamide 5 x SCC, 2 x Denhardt's solution (Sambrook, Fritsch, Maniatis Molecular  
20 Cloning- A Laboratory Manual 2nd Ed. 1989), 0.1% SDS and 100 ug/ml salmon testes DNA) for 2 hours at 32°C. The filters were then hybridized overnight in a shaking water bath at 42°C with the labeled probe.

The filters were washed with 2 x SSC/0.2% SDS at 65°C until it was determined that there was minimal radioactivity coming off in the wash solution. The filters were then blotted  
25 dry and exposed to X-ray film (Kodak XAR) (Kodak, Rochester, New York) with image intensifying screens at -80° C overnight.

A total of 38 duplicating positive plaques were picked and eluted into 1 ml of SM Buffer (0.1M NaCL, 0.008M MgSO<sub>4</sub> 7H<sub>2</sub>O, 0.05M Tris-HCl [ph 7.5], 0.01 gelatin.) Fifteen of the positives were then plated out (0.5 ml of a 10<sup>-4</sup> dilution), and used for a second round of screening, using the same hybridization/wash protocol as above. A single, well isolated hybridizing plaque was picked from each of the 15 positives and eluted into 1 ml SM solution. The phages were rescued into pBluescript (Lambda Zap II) using the ExAssist/SOLR System (Stratagene). Ampicillin resistant colonies were obtained from ten of the fifteen second round positive picks.

The subcloning process went as follows. The phagemid DNA obtained for the library screening process was labeled pSyn23/1. pSyn23/1 was double digested with the restriction enzymes Eco RI and Cla I (All restriction primers enzymes are available from Promega, Madison, Wisconsin) to produce a 3 kb fragment. The isolated fragment was ligated into pBluescript II (Stratagene, La Jolla, CA) and transformed into DH5alpha, (Stratagene) giving pSyn23/1-I. This AHAS clone was sequenced using the fmol DNA Sequencing System (Promega, Madison, Wisconsin) and a set of eight gene-specific primers plus the T3 sequencing primer located in the pBluescript II vector. An open reading frame of 625 amino acids was identified.

The resulting sequence of large subunit *ilvG*, identified as SEQ ID NO:6, had a sequence as follows:

**Acetohydroxy Acid Synthase (*ilvG* gene ORF)**

>Synechocystis sp. strain PCC6803

```
GCCATAGGAGCCCATCGCCGATTGAGTTCAAATTAGAAGCACTTAGCCTACGCTT
CCTAAACCGATTGTCCAGTGGTTGCATCAATTCCTAATCCCAAACAAATTCCT
GAAAACTGTTCTAGCCAACGGCAAACCGGGGCTTATATCCTGATGGATAGCCTG
AAACGCCATGGGGTCAAACACATTTTGGCTATCCCGGCGGGGCAATTTGCCCA
TCTATGATGAACTGTACCGCTTTGAAGCGGCGGGGAAATTGAGCATATTTGGT
GCGCCATGAACAAGGAGCTTCCCATGCGGCGGATGGGTATGCCAGAGCCACAGG
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TAAAGTGGGAGTTTGTTCGGTACATCTGGACCAGGGGCGACTAACTTGGTGACC  
GGCATTGCCAATGCCCATTTGGACTCGGTGCCCATGGTGGTGATTACTGGAGAGG  
TGGGCCGTGCCATGATTGGTAGCGATGCTTTCAGGAAATTGACATTTTTGGCAT  
CACCTTACCGATCGTTAAGCACTCCTATGTGGTACGTAGTGCGGCGGATATGGCT  
5 CGCATTGTTACTGAGGCTTTCATCTTGCTAGCACCGGTCGTCCCGGGCCGGTTTT  
GATCGATATTCCCAAGGATGTGGGCTTAGAAGAATGTGAGTACATTCCCCTCGAC  
CCCGGTGACGTTAATCTACCGGGTTATCGCCCCACGGTTAAAGGTAATCCCCGAC  
AAATTAATGCGGCATTGCAATTGTTGGAGCAGGCCAGAAATCCCTTGCTCTACGT  
AGGGGGAGGGGCGATCGCCGCCAATGCCCATGCCAGGTGCAGGAATTTGCGGA  
10 AAGGTTCCAGTTGCCGGTAACAACCACCCTGATGGGAATTGGGGCTTTTGACGAA  
AACCATCCCCTTTCGGTGGGTATGTTGGGTATGCATGGCCACCGCTATGCCAACT  
TTGCCGTCAGCGAATGTGATTTGTTGATTGCAGTGGGGGGCCCGTTTCGACGACCG  
GGTAACTGGCAAACCTAGACGAATTTGCTAGCCGCGCCAAAGTAATTCACATTGAC  
ATCGACCCGGCGGAGGTGGGAAAAAACAGGGCTCCCGATGTGCCCATTTGTGGGG  
15 GATGTACGCCATGTTTTAGAACAGCTTTTGCAGCGGGCCCGGGAATTGGATTACC  
CCACCCATCCCCATACCACCCAGGCATGGTTAAATCGCATTGATCATTGGCGGAC  
CGATTACCCCCTCCAGGTGCCCCACTATGAGGATACTATTGCCCCCAGGAGGTA  
GTACACGAAATTGGTCGCCAGGCCCCCGATGCCTACTACACCACCGATGTGGGAC  
AACACCAAATGTGGGCGGCCAGTTTTTGAACAATGGCCCCCGCCGATGGATTTC  
20 CAGTGCTGGCTTGGGTACGATGGGCTTTGGTTTACCTGCCGCCATGGGAGCCAAA  
GTGGGAGTGGGGGACGAGCGGTCATTTGCATCAGTGGAGATGCCAGCTTCCAAA  
TGAATCTTCAGGAACTGGGAACCCTAGCCCAGTACGACATCCAGGTAAAACTAT  
TATTCTCAATAACGGTTGGCAGGGGATGGTGCGTCAGTGGCAACAACTTTCTAC  
GAAGAACGTTATTCTGCTTCTAACATGTCCCAGGGCATGCCAGACATTAATCTCC  
25 TCTGTGAAGCCTATGGCATCAAGGGTATTACTGTGCGCAAGCGGGAAGATTTGGC  
CCCGGCGATCGCCGAAATGCTAGCCCACAATGGTCCTGTGGTGATGGATGTGGTG  
GTCAAAAAAGATGAAAACGTGTTACCCTATGATTGCCCCCGGCATGAGTAATGCCC

AAATGCTAGGTTTACCGGAAGTGCCGGTACNGGACAATGGTCCCCGGATGGTGG  
 AGTGCAACCATTGCCAAACCCAAAATTTTCATCACCCATCGTTTCTGTTCTGGTTGT  
 GGAGCCAAACTCTAACCATAAGCCAAAATTGAATTC

5 The predicted amino acid sequence of the open reading frame had 49% identity to the  
*E. coli* ilvG AHAS gene, 47% identity to the maize *als2* gene, 46% identity to the  
*Arabidopsis* AHAS gene, and 65% identity to the sequence the AHAS gene from the  
 cyanobacterium *Spirulina platensis*. The high degree of sequence identity and the functional  
 demonstration of the cyanobacterial gene fragment in complementing the AHAS deficient *E.*  
 10 *coli* mutants strongly suggest that the fragment represents a full length cyanobacterial AHAS  
 large subunit gene.

To confirm that these plasmids carry functional AHAS sequences, plasmid DNA  
 from each of the ten rescued colonies was transformed into the *E. coli* strain M1262. (leuB6,  
 ilvI614, ilvH612,  $\lambda^-$ , relA1, spoT1, ilvB619, ilvG603, ilvG605 (am), thi-1) (Genetics Stock  
 15 Center, Yale University). This strain of *E. coli* is lacking in AHAS. Three of the plasmids  
 were found to enable growth on M9 (+Leu) plates, thus indicating that these plasmids carried  
 functional AHAS copies. *E. coli* M1262 expressing the cyanobacterial *ahas* gene were  
 capable of growing on minimal media in the presence of OUST® sulfometuron methyl and  
 PURSUIT® imazethapyr herbicides. The *ahas* gene can therefore be used for achieving  
 20 herbicide tolerance in crops by transformation into the nuclear or plastidic genome.

#### **Example 7: Cloning and sequencing the Large *Synechocystis* AHAS Gene**

The phagemid DNA from one of the complementing lines pSyn23/1 was doubly digested with  
 25 the restriction enzymes Eco RI and Cla I (Promega) to produce a 3 kb fragment. The Eco RI  
 and Cla I were excised out of the pBluescript phagemid as the The isolated fragment was  
 ligated into pBluescript and transformed into DH5alpha (Stratagene), creating pSyn23/1\_I.

The resulting AHAS clone was sequenced using the fmol DNA Sequencing System (Promega) and a set of eight gene-specific sequencing primers:

SYN1: 5' ATT GAC ATT TTT GGC ATC 3', identified as SEQ ID NO: 7

5 SYN2: 5' TAT CCG CCG CAC TAC GTA C 3', identified as SEQ ID NO: 8

SYN3: 5' CAG GGG CGA CTA ACT TGG TGA C 3', identified as SEQ ID NO: 9

SYN4: 5' ACC GCT ATG CCA ACT TTG CCG T 3, identified as SEQ ID NO: 10

SYN5: 5' GGA GGA TAG TAC ACG AAA TTG G 3', identified as SEQ ID NO: 11

SYN6: 5' AAA TCT TCC CGC TTG CGC ACA G 3', identified as SEQ ID NO: 12

10 SYN7: 5' CCA ATT TCG TGT ACT ACC TCC TG 3', identified as SEQ ID NO: 13

SYN8: 5' AAA GTG GGA GTG GGG GAC GAA 3', identified as SEQ ID NO: 14

Additionally, a T3 sequencing primer located in the pBluescript II vector was added.

15 An open reading frame (ORF) of 635 amino acid was identified. The predicted amino acid sequence of the open reading frame had 49% identity of the *E. coli ilvG AHAS* gene, 47% identity to the maize *als2* gene, 46% identity to the *Arabidopsis AHAS* gene, and 65% identity to the sequence of the *AHAS* gene from the cyanobacterium *S. plantensis*.

#### Cloning of the AHAS small subunit from *Synechocystis*

20 In another embodiment of the present invention, a *Synechocystis* AHAS Small Subunit nucleic acid fragment was also cloned from a genomic DNA library of cyanobacterium *Synechocystis* PCC 6803.

Database searches of the complete genomic sequence of *Synechocystis* revealed three different ORFs encoding genes of acetolactate synthase, *ilvG*, *ilvB*, and *ilvN*. Further  
25 sequence similarity comparisons suggested that *ilvN* is likely to encode the small subunit of *Synechocystis* AHAS. To clone *ilvN* from *Synechocystis*, a PCR-based approach was adopted. Based on the sequence data, a pair of primers with the following sequences were

designed, primer #1 (forward primer): 5'-cggtggaattttacccaatgg-3', identified as SEQ ID NO: 15 and primer #2 (reverse primer): 5'-ggccctaaaacttgattccagg-3', identified as SEQ ID NO: 16 and these primers were used to PCR amplify the corresponding ORF (*ilvN*) from genomic DNA prepared from wild type cell cultures of *Synechocystis*.

Agarose gel analysis of PCR products yielded a band with the expected size (573 bp). PCR products have subsequently been subcloned into the Invitrogen TOPO pCR2.1 TA vector.

The gene was sequenced using the same procedures as above.

The resultant *Synechocystis* sp. strain PCC6803 revealed the sequence, identified as

SEQ ID NO: 17:

GTGGAATTTTACCCCAATGGCCACCGGCGATCGCCTTCTTTGCCCCCATGAAAC  
ACACCCTCTCTGTTTTAGTTGAAGATGAAGCCGGAGTGCTAACCCGCATTGCCGG  
ACTATTGCCCCGCCGTGGTTTTAACATTGAGAGCTTGGCGGTGGGGTCGGCGGAA  
CAGGGGGACGTTTCCCGCATCACCATGGTGGTGCCGGGGGATGAGAACACCATC  
GAACAACTGACCAAGCAACTCTACAAGTTGGTTAACGTAATTAAAGTACAGGAC  
ATCACCGAAACTCCCTGTGTGGAAAGGGAATTGATGCTGGTGAAGGTGAGCGCC  
AATGCCCTAACCGAGCGGAAGTGATTGAGCTAGCCCAGGTATTCCGGGCCCCGC  
ATTGTGGATATCTCCGAAGACACCGTCACCATCGAATGGTGGGGGACCCGGGTA  
AAATGGTAGCAATCCTCCAGATGTTGGCCAAGTTGGCATTAAAGAGGTGGCTCGA  
ACGGGCAAAATTGCTTTGGTGCGGGAATCCGGCGTCAATACGGAATATCTGAAAT  
CCCTGGAATCCAAGTTTTAG

#### Construction of a nuclear plant transformation vector

Transformation of the *AHAS* genes into the nuclear genome required a nuclear plant transformation vector. Since branched chain amino acid biosynthesis is localized in the chloroplast in higher plants, for functional expression of *AHAS* in higher plants, the prokaryotic *Synechocystis* *AHAS* large subunit gene would need to be both expressed off of a

plant expressible promoter and the protein would need to be targeted into the chloroplast. Therefore, a leader peptide will have to be fused onto the *Synechocystis* AHAS for it to be functional in the nuclear genome. When the gene is imported into the chloroplast, the leader peptide gets clipped. The final result would be the *Synechocystis* AHAS gene within the chloroplast minus the transit sequence.

Because the *Synechocystis* AHAS lacks the leader or transit protein sequence required to be active in the nuclear genome and transported into the chloroplast, the promotor and transit sequence of another organism was fused with the *Synechocystis* AHAS gene.

The promoter and transit sequence from the *Arabidopsis* AHAS large subunit was chosen to be fused to the *Synechocystis* AHAS large subunit gene, as there was a large degree of homology. The *Arabidopsis* genome has been sequenced and the physical and sequence information for AHAS large subunit can be found at

<http://www.arabidopsis.org/servlets/mapper?value=CSRI&action=search>. One skilled in the art could use the information at this database to perform the cloning as follows. The final result would contain the promotor and transit sequence of the *Arabidopsis* AHAS gene, followed by the *Synechocystis* gene, followed by the *Arabidopsis* terminator. The source of the promoter and transit sequence was the construct pAC793, (which consisted of a vector and an insert with a genomic fragment containing the *Arabidopsis* AHAS promoter, transit sequence, coding region, and terminator.)

An alignment of the *Synechocystis* and *Arabidopsis* AHAS large subunits was made using the Gap program from Genetics Computer Group Inc. (GCG, Inc., Madison, Wisconsin). A region of homology near the N-terminal of the *Synechocystis* AHAS gene and past a putative transit sequence processing site on the *Arabidopsis* AHAS gene was chosen to make a fusion between the *Arabidopsis* transit sequence and *Synechocystis* AHAS. A common Eco RV restriction site in both the *Arabidopsis* and *Synechocystis* AHAS gene that was within a conserved region of the proteins was used as the fusion site. An Age I restriction site occurs naturally in the *Arabidopsis* gene. The site was found to be past the



processing site and just past the stop codon of the *Arabidopsis* AHAS gene. Thus, it was chosen to create a fusion between the C-terminal end of the *Synechocystis* AHAS gene and the *Arabidopsis* AHAS termination sequence by insertion of an Age I site in the *Synechocystis* gene in a region homologous with the *Arabidopsis* gene.

5           PCR primers were designed to insert an Age I (primer SYNAGE) restriction sites on the 3-prime end of the *Synechocystis* AHAS gene. pAC793, a construct cloned from *Arabidopsis* abd contains genomic AHAS in a pGEM vector (Promega), was cut with Eco RV and Age I to remove most of the coding sequence of the *Arabidopsis* AHAS gene from the vector. The construct pSyn 23/1\_I which contain a subcloned genomic fragment from  
10   *Synechocystis* (an Eco RI - Cla I subclone from the plasmid pSyn 23/1. pSyn23/1 was the resulting plasmid from screening the *Synechocystis* genomic library, first paragraph of this section. PSyn 23/1-1 was created by digesting pSyn 23/1 with Eco RI and Cla I and purifying the resulting fragment. The fragment was then ligated into pBluescript II that had been previously cut with Eco RI and Cla I.) that contained the entire AHAS gene was cut with Nco  
15   I and Age I to confirm that the correct fragment was obtained.

          Using the pSYN 23/1\_I vector as a template a PCR reaction was carried out with the primers. The reactions gave an expected 1.9 kb PCR fragment when run out on a 0.8% TAE agarose gel. The fragment was cloned into the TA cloning vector (TA cloning kit, Invitrogen) using a Ready-To-Go ligation vial (Pharmacia). The ligation products were transformed into  
20   competent cells from the TA cloning kit (Invitrogen).

          The cells were gently transferred SOC media (Qbiogene, Carlsbad, California) then gently transferred to a sterile culture tube and incubated. The cells were then plated on a blue-white media and incubated overnight at 37°C. The following day white colonies were selected.

25           Plasmid minipreps were made from cultures of selected white colonies. Restriction digestion of the plasmids generated expected fragments on agarose gels. The construct containing the fusion of the *Arabidopsis* AHAS large subunit promoter and transit sequence,

the *Synechocystis* AHAS large subunit coding region, and the *Arabidopsis* AHAS large subunit termination sequence in the pGEM vector of the pAC793 vector, was labeled pGEKI. This construct could then be used for nuclear genome transformation where the *Synechocystis* AHAS gene is to be transported from the genome into the chloroplast.

5

#### **Example 8: Creation of a Nuclear Plant Transformation Vector**

A nuclear plant transformation vector was constructed as follows. PCR primers were designed to insert Eco RV (primer SYN5) and Age I (primer SYNAGE) restriction sites on the 5-prime and 3-prime ends, respectively, of the *Synechocystis* AHAS gene. They are identified as Sequence ID No. 18 (SYN5) and Sequence ID No. 19 (SYNAGE). pAC793 was cut with Eco RV (just past the transit sequence) and Age I (just past the stop codon) to remove most of the coding sequence of the *Arabidopsis* AHAS gene from the vector. The remaining 7 kb fragment containing the pGEM vector, the *Arabidopsis* AHAS promoter, the transit sequence and the termination sequence was removed from an agarose gel and treated with phenol:chloroform:isoamyl alcohol washes. The fragment was cut again with Eco RV and Age I to make sure restriction digests were complete. The construct pSyn 23/1\_I was obtained that contained a subcloned genomic fragment from *Synechocystis* (an Eco RI - Cla I subclone from the plasmid pSyn 23/1) which in turn contained the entire AHAS gene cut with Nco I and Age I to confirm that the correct fragment was obtained.

PCR primers were designed to insert an Age I (primer SYNAGE) restriction site on 3-prime end of the *Synechocystis* AHAS gene. A 5 prime primer was designed to amplify the gene upstream of the Eco RV site.

SYN5: 5'-GGC TGA TAT CCT GAT GGA TAG CCT G-3', identified as Sequence ID No.

18

SYNAGE: 5'-TTG GCT TAC CGG TTA GAG TTT GGC TCC ACA-3', identified as  
Sequence ID No. 19.

Using the pSYN 23/1\_I vector as a template, a PCR reaction was carried out with the  
5 primers. The reactions (35 cycles of 94°C melting, 55°C annealing and 72°C polymerase  
elongation (Perkin Elmer Thermocycler) gave an expected 1.9 kb PCR fragment when run out  
on a 0.8% TAE agarose gel.

Two uL of the PCR reaction was diluted 8X. The TA cloning vector (TA cloning kit,  
Invitrogen) was resuspended in 8.8 ul of TE. (Tris/EDTA. Sambrook, Fritsch, Maniatis  
10 Molecular Cloning- A Laboratory Manual 2nd Ed.1989). Two uL of TA cloning vector was  
added to a ligation vial (Ready-To-Go, Pharmacia). Additionally, one uL of 8X diluted PCR  
amplified fragment was added to the solution. Sterile water was added to bring the volume up  
to 20 uL. Without mixing, the vial was kept at room temperature for five minutes. After 5  
minutes, the solution was mixed gently by sucking the solution in and out of a pipette tip. The  
15 sample was briefly spun to bring the solution to the bottom of the tube. The vial was then  
placed in a 16°C water bath for 45 minutes.

Two uL of Beta-mercaptoethanol was added to each vial of competent cells provided  
in the Invitrogen TA cloning kit (Invitrogen). After 45 minutes in the ligation reaction, the  
vials were placed in ice for 3 minutes. Two uL of the ligation mix were added to the  
20 competent cells. The vial were then incubated on ice for 30 more minutes, followed by 60  
seconds of heat shock at 42°C. The vials were again placed on ice for 3 minutes.

The cells were gently transferred to 450 µL of room temperature SOC media  
(Qbiogene, Carlsbad, California) then gently transferred to a sterile culture tube and incubated  
by an hour of shaking at 225 RPM at 37°C. The cells were then plated on LB/amp/X-gal  
25 (Sigma) (Sambrook, Fritsch, Maniatis Molecular Cloning- A Laboratory Manual 2nd Ed.  
1989) plates and incubated overnight at 37°C. The following day white colonies were  
selected.

Plasmid minipreps were made from cultures of selected white colonies. Restriction digestion of the plasmids generated expected fragments on agarose gels.

The construct containing the fusion of the *Arabidopsis* AHAS large subunit termination sequence in the pGEM vector was labeled pGEK1.

5

### Nuclear Transformation of Cyanobacterial Genes into Plants

#### *Agrobacterium* vector construction

Tobacco plants were transformed with the *Arabidopsis/Synechocystis* AHAS fused gene. The vector pGEK1 was cut with Kpn I and Sal I to remove the entire AHAS fused gene from the PGEM vector and was ligated into a pBIN19 *Agrobacterium* vector (Stratagene) that was previously cut with the same enzymes. Restriction analysis indicated that the fusion gene from pGEK1 was successfully moved into the plant transformation vector.

10

Plants were selected on 100 mg/L kanamycin. Tobacco cultivar, *Wisconsin-38* (North Carolina State University, US Tobacco Germplasm Collection) was grown aseptically on MSh<sup>-</sup> medium (Sigma) containing sucrose (20g/L) in glass (1qt.) jars. Stem segments from plants 8-10 week were transferred to new jars for leaf propagation. Total DNA was extracted from tobacco lines using the Qiagen DNeasy Miniprep kit. (Qiagen, Inc., Valencia, California).

15

Tests showed the transformants had little resistance to imidazolinone herbicides. This may have been due to several reasons. One reason may be that the *Synechocystis* AHAS large subunit was not accompanied by an AHAS small subunit. It has been shown that microbial AHAS genes are comprised of a large and small subunit. The large subunit of AHAS from *E. coli* does not have optimal activity in the absence of the corresponding small subunit. Since *Synechocystis*, similar to *E. coli*, is a prokaryotic organism it may share the same requirement. The absence of the small subunit may have diminished the activity of the enzyme and the ability to confer imidazolinone resistance.

20

25

Another potential reason for lack of resistance may have been the selection of the position of the fusion junction between the *Arabidopsis* AHAS transit sequence and the *Synechocystis* large subunit. An improper fusion junction may have produced a protein that either could not be localized in the chloroplast or produced a non-functional protein.

5

### Plastid Transformation

It is believed that chloroplasts in higher plants were derived from cyanobacteria. The ancestral relationship between chloroplasts and cyanobacteria suggests that genes, gene elements, proteins, and many other features of the organisms are similar and potentially cross-functional. Cyanobacterial genes and gene elements may therefore be functional when transformed into plastid genomes. Moreover, expression of proteins from plastidic genomes obviates the need for transit sequences to traffic the protein to the proper location.

Therefore, use of cyanobacterial genes, or mutant genes isolated from resistant strains, for achieving herbicide resistance can be obtained by transformation into the plastome. Transgenes from alternative sources will confer different characteristics of the expressed traits. Regulatory elements of cyanobacterial genes can be used for control of expression in plastids. If a transgene is located in the plastome of a crop, its transfer to related species (weeds and/or crops) via pollination is prevented. The transgene will be expressed from a high number of copies per cell suggesting very high levels of expression.

Furthermore, The location of transgene in the plastome obviates transport of gene products into the plastids and cyanobacterial genes can be used without modification of the coding region.

Thus, in preferred embodiments this invention provides cyanobacteria as an alternative source of genes for plant transformations, in particular genes encoding herbicide insensitive proteins, and elements of genes for control of expression in plastids. Furthermore, since sequenced DNA fragments contain prokaryotic regulatory elements, cyanobacteria can be directly used for plastome targeted transformations.

Specifically, the *Synechocystis* AHAS large subunit gene was used for transformation into plant chloroplasts to confer herbicide resistance.

#### Plastidic Transformation of Cyanobacterial Genes into Plant Chloroplasts

5           The genes were constructed into vectors to permit incorporation into and expression in the chloroplasts. The following vectors were constructed for transformation into plastid genomes. pACBC111 and pACBC112 are related constructs differing only in the orientation of the *Synechocystis* AHAS expression cassette. These vectors were constructed as shown in **Figures 11 and 12**. The aadA sequences and the p16S expression cassette are derived from  
10           the sequences described in U.S. Patent No. 5,877,402 (Maliga et al.). The disclosure of this patent is incorporated by reference herein in its entirety. pACBC 111 is the same vector as p12 delta NI. pACBC112 is the same vector as p12 delta NII. p116 I (**Figure 13**) is the same as pACBC222 and p116 II (**Figure 14**) is the same as pACBC223. pACBC111 (or p12 delta NI) and pACBC112 (or p12 delta NII) are constructs where the *Synechocystis* AHAS gene  
15           and the aadA gene are expressed from individual promoters (**Figures 11 and 12**) The p116 I and p116 II are discistronic constructs, one promoter expressing an operon with two genes (Syn AHAS and aadA) differ from each other only in the orientation of the *Synechocystis* AHAS expression cassette (**Figure 15**). The 222 and 223 vectors and the 111 and 112 vectors differ in that the p222/223 constructs are designed to express a dicistronic message while the  
20           p111/112 constructs will express the gene from a monocistronic insert.

#### Transformation and regeneration of transplastomic plants

Plasmids pACBC222, pACBC111 and pACBC112 were used for plastid transformations. Leaves were cut and placed abaxial side down on regeneration medium  
25           (Msh<sup>-</sup> medium supplemented with zeatin (2mg/L), 1-naphthaleneacetic acid (0.1mg/L), and sucrose (20g/L).

Bombardments were carried out using the DuPont PDS 1000He Biolistic gun. (DuPont, Wilmington, Delaware). Rupture discs (900psi) (BioRad, Hercules, California) were used, and helium pressure and vacuum levels were 1100psi and 27" Hg, respectively.

Two days after bombardment, leaves were cut into 1 cm<sup>2</sup> pieces and placed on Spectinomycin (500 mg/L). Expanding and regeneration leaf segments were passed for up to 4 rounds on selection media. Fourth round regenerates were transferred to Magenta boxes (Sigma, St. Louis, MO) until sufficient roots were exhibited to warrant transplantation to the greenhouse.

#### **Example 9: Plastid Transformation**

Plasmids p116, p12delta NI and p12delta NII were used for plastid transformations in the transformation and regeneration of transplastomic plants. Leaves were cut and placed abaxial side sown on regeneration medium (Msh<sup>-</sup> medium supplemented with zeatin (2mg/L) (Sigma), 1-naphthaleneacetic acid (0.1mg/L), and sucrose (20g/L). Gold was prepared for transformation by weighing 5mg of gold (0.6um) into Treff tubes (Treff AG, Degersheim, Switzerland) and washing once with both ETOH (100%) and sterile bidistilled water. The gold was pelleted and re-suspended in 230 uL water and mixed with 20ug DNA (p116, p12delta NI, or p12delta NII), 250uL CaCl<sub>2</sub> (2.5M), and 50uL spermidine (Sigma) (0.1M free base). The gold/DNA mixture is then incubated on ice for 10 minutes and centrifuged. Two ETOH (100%) washes were performed, and the gold/DNA was suspended in 72uL ETOH (100%). The gold suspension (5.4ul) was applied to each macrocarrier (BioRad). The macrocarriers were then placed in a dessicator for at least 1 minute.

Bombardments were carried out using the DuPont PDS 1000He Biolistic gun. Rupture disks (900psi) were used, and helium pressure and vacuum levels were 1100psi and 27" Hg, respectively. Two days later, leaves were cut into 1cm<sup>2</sup> pieces and places on selective regeneration medium containing spectinomycin (500 mg/L). Leaf segments from the first

round regenerates were taken and placed on the same medium. Leaf segments were then taken from the second round regenerants and placed on two parallel selection plates. One regeneration medium contained only 500 mg/L of spectinomycin, and the other regeneration medium contained both 500 mg/L of spectinomycin and 500 mg/L of streptomycin. Leaf segments that remained green and showed signs of callus formation or regeneration on the dual selection media were selected and placed in a regeneration medium that contained only spectinomycin for a third round of regeneration.. Regenerants were transferred to Magenta boxes (Sigma, St. Louis, MO) until sufficient roots were grown to warrant transplantation to a greenhouse.

#### **E. Selectable Resistance Marker for Transformations**

The present invention, in addition, includes the use of the cyanobacterial *pds* and *ahas* genes as a selectable marker for transformations. To test the ability of *pds* and *ahas* genes as selectable markers, *aadA*, a known marker for streptomycin and spectinomycin was used as a control. Upon transformation, a plant transformed with *pds* or *ahas* and *aadA* should show resistance to streptomycin, spectinomycin as well as the imidazolinones or 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide. In this instance, *aadA*, a known marker for streptomycin and spectinomycin, was used as a control. Thus, a plant grown with *pds* or *ahas* and *aadA* should show resistance to streptomycin, spectinomycin and imidazolinones or 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide.

To test for cyanobacterial *pds* and *ahas*'s ability as selectable markers, leaf explants were transferred to medium containing both spectinomycin and streptomycin following two rounds of regeneration under spectinomycin selection. The numbers of spectinomycin/streptomycin resistant lines for each construct can be seen on **Table 3**.



**Table 3.** In vitro selection of plastid transformants.

DNA Construct	# of Bombardments	# of Discrete spectinomycin-resistant Lines	# of spectinomycin + streptomycin resistant lines
p12AN I	35	6	3
p12AN II	35	5	3
p116	90	12	1

Observations and photos (**Figure 8**) of the PURSUIT® imazethapyr spray test were taken 5 weeks after the test was conducted. Wild type (W-38), p 111, and p 112 lines showed wide-spread leaf necrosis and stunting of growth when sprayed at an 18g/ha concentration, and even more extreme effects were seen at 35g/ha. p116 line. G-981208-1.1, showed no visible leaf damage at 18 or 35g/ha. Growth was uninhibited at 18g/ha, although slight stunting could be observed at 35g/ha. PURSUIT® imazethapyr appeared to act as a strong growth regulator on the p116 line, resulting in prolific shooting and morphological abnormalities in new shoots. Leaves assumed a thin, spiny form.

PCR amplification confirmed the integration of the *Synechocystis AHAS* gene into transplastomic line G-981208-1.1 (a-d) (**Figures 9A and 9B**). Clone *a* was sprayed at 35g/ha PURSUIT® imazethapyr, clones *b* and *c* were sprayed at 18g/ha, and clone *d* was not sprayed. The properly sized bands could be seen for the *AHAS* fragment.

Therefore, the *ahas* gene successfully integrated into the plastome and provided herbicide resistance. Because of this, cyanobacterial *pds* and *ahas* mutants can be used as a control selectable markers to test other types of transformations. The herbicide resistant *pds* and *ahas* genes can be coupled with selection on 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide or other known PDS inhibitors, and imidazolinones and other AHAS inhibiting compounds such as PURSUIT® imazethapyr for an efficient selection system for

transformation. Selections can be applied to either nuclear or plastid transformation, depending on the construction of the genes.

The pACBC222 line, G-981208-1.1, tobacco cultivar, *Wisconsin-38* transformed with the pACBC22 (or p116 I) construct (**Figure 10**) sprayed with 18g/ha PURSUIT® imazethapyr showed about a 20% increase in *AHAS* enzyme resistance in the presence of PURSUIT® imazethapyr and sulfanylcarboxamides when compared with *AHAS* enzyme from unsprayed wild type tobacco. Interestingly enough, it appears that the *snAHAS* enzyme displays a level of cross resistance to both PURSUIT® imazethapyr and sulfanylcarboxamides, although they are both quite dissimilar structurally.

#### **F. Cells, Tissue, Plants derived from Chloroplast-mediated Transformations**

A further object of this invention provides for cells, tissue, plants, pollen derived from said transformation of the mutant *Synechocystis pds* gene and the *ahas* genes into untransformed plant cells, using . Alternatively, mutant forms of *pds* genes with mutation(s) at position(s) similar to the *Synechocystis* gene can be obtained for any given crop species, and used further for genetic transformation. *Synechocystis* mutant *pds* gene(s) resistant to 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide and the mutant *AHAS* gene comprising the *ahas* small subunit and the *ahas* large subunit identified in these processes can be, respectively, introduced directly into crops for engineering 4'-fluoro-6-[(alpha,alpha,alpha,-trifluoro-m-tolyl)oxy]-picolinamide resistance via chloroplast-mediated transformation and imidazolinone resistance. The genes can also be used for generating resistance to other *pds* or *AHAS* inhibiting herbicides.

While the preferred embodiments of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

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